PERSPECTIVE ARTICLE

Next-generation strategies to improve safety and efficacy of adeno-associated virus-based gene therapy for hemophilia: lessons from clinical trials in other gene therapies

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Abstract

Three major directions for the global progress of adeno-associated virus (AAV) vectors for gene therapies (GT) are analyzed: 1) engineering vectors to increase transgene expression; 2) aligning interests of the health system with costs and challenges for the pharmaceutical industry; and 3) refining patient eligibility criteria and endpoint definition. Currently employed AAV vectors may cause toxicity and adverse events. Furthermore, studies in animals do not fully predict risks and clinical benefits of AAV-based GT, and animal models reflecting the heterogeneity of certain clinical settings (e.g., congestive heart failure) are not widely available for improving AAV-based GT. Finally, antisense and gene editing approaches will soon complement gene augmentation strategies for the stable solution of unsolved issues of AAV-based GT. While minimizing toxicity, next-generation AAV vectors should decrease the viral load needed to achieve therapeutic efficacy, be functional in a restricted cellular subset, avoid transgene expression in unwanted cells (e.g., hepatocytes), and escape immune oversight in AAV-based GT. The role of stress-induced apoptosis in the loss of transgene expression in GT should also be explored. Aligning the interests and obligations of the pharmaceutical industry with those of the health system is critical for the success of AAV-based GT. Costs and challenges for the pharmaceutical industry include: a) removing impurities from AAV; b) validating tests to measure treatment efficacy; c) promoting training programs to standardize vector genome delivery; d) collecting long-term follow-up data; and e) maintaining sustainability and cost-effectiveness of AAV-based GT. In rare disorders with small patient numbers (e.g., hemophilia), clear-cut outcomes are mandatory as endpoints of unequivocal efficacy data.

Introduction

At the end of the last millennium, unfortunate events virtually paused gene therapy (GT) studies.[1,2] Huge progress has been made since then to develop more proficient and safer viral vectors. Self-replicating RNA viruses have proven excellent for vaccine and cancer therapy (high-level, short-term transgene expression). For their long-term transgene expression, adeno-associated virus (AAV) and lentiviral vectors have been preferred for GT studies in inherited or chronic diseases.[3] AAV have higher biosafety compared to lentiviral vectors,[4,5] and are easy to use because no patient treatment is needed prior to injection / infusion, and the vector is injected locally (e.g., subretinal) or infused *in vivo* (via a peripheral vein or via a catheter for intrathecal infusion). This has meant that second-generation AAV-based GT have been widely applied in studies for ophthalmological, hematologic, immunological, muscular, neurological, metabolic, and cardiovascular diseases. Hence, after more than 30 years of research, development, and early clinical reports in the field of gene and cell therapy, 32 advanced therapeutic medicinal products (ATMP), based on cell or gene therapy sometimes in combination (the definition of the European Medicines Agency, EMA), and finalized for use in inherited and malignant diseases have been approved for commercialization in Europe (EU) and the United States (US). Eleven of them, devoted to single gene hematologic and non-hematologic diseases, provide

new options for severe clinical conditions, including some that were previously considered untreatable.[6] The number of related publications is increasing exponentially. In addition to scientific and regulatory advice to researchers and clinicians, and information to manufacturers, evidence on safety limits, hopes and concerns relative to their conversion to fully viable and safe medicines have emerged. Major lessons from these publications may be critical to improve AAV-based GT, whose global progress is the thrust of this review.

The landscape of clinical trials in adeno-associated virus-based gene therapies

A survey covering over 140 clinical trials of AAV-based gene therapies and involving more than 3,000 patients treated for more than 20 years showed that AAV-based GT is a well-tolerated and effective treatment modality.[7] In 21% of these trials, low-grade adverse events (AE) were detected within the first month after AAV administration, and 35% of them were accounted for by vector components. Increase in alanine aminotransferase (ALT) levels, occurring in high dose (>10^{13} vector genomes per kg of body weight [vg/kg]) systemically (mostly intravenous, IV) administered cohorts, was first detected in persons with severe hemophilia B (HB) undergoing GT.[8] No preclinical model helped to predict this hepatotoxic event (credited to vector and transgene-specific T-cell responses directed against AAV-transduced hepatocytes), that showed a positive response to corticosteroid administration.[8] Liver toxicity is dose-dependent and was more severe in infants with spinal muscular atrophy 1 (SMA) receiving higher doses of systemically delivered self-complementary AAV9 in order to achieve high-level neuronal transduction and positive effects on neuromuscular transmission and growth.[9] On day 9 after gene delivery, ALT levels increased 16 times the upper limit of normal range in the first treated patient. The US Food and Drug Administration (FDA) approved a protocol amendment that introduced prednisone (1 mg/kg/d started 24 hours [hr] prior to gene delivery) and reduced viral load from 3.3x10^{14} to 2.0x10^{14} vg/kg for the high-dose cohort.[10] Asymptomatic elevations of serum ALT levels occurred in 3 children, all reversed with oral steroids.[10] Compared with natural history controls, obvious improvement in overall survival, motor function and motor milestones was documented in the children enrolled in the trial.[10] Major AE (Table 1) were also documented in AA-based GT. In spite of substantial improvements in daily hours of ventilator dependence and in motor function, 4 children with severe X-linked myotubular myopathy (XLMTM) receiving resamirigene bilparvovec died after receiving GT.[11] All had cholestatic liver failure at the time of death (Table 1). While the 3x10^{14} vg/kg dose of AAV vector copies is among the highest ever used in humans, the interpretation of these deaths is complicated by a previously unknown tendency for cholestatic liver disease in children with XLMTM. These serious AE were unlikely to be related to immune responses: none of the 4 participants (all with severe liver injury) received any benefit from prophylactic doses of prednisolone and, in some cases, high-dose methylprednisolone and other immune-modulating therapies. Thrombocytopenia, hemolytic anemia, acute kidney injury, microvascular thrombosis, abnormal structure of von Willebrand factor and dysregulation of the alternative pathway of complement are common findings in acquired thrombotic microangiopathy (TMA).[12] TMA has been reported following systemic AAV9-based GT for SMA, for Duchenne Muscular Dystrophy (DMD) or for Dannon disease, and of AAV-LK03 GT for methylmalonic acidemia (MMA).

Supporting the possibility of an immune-mediated etiology, TMA developed approximately one week after the AAV-based delivery of a copy of Zolgensma® in the children with SMA.[13] In some of them, the clinical history revealed triggers (vomiting, and/or infections with encapsulated organisms) prior to developing TMA. Eculizumab, plasmapheresis, corticosteroids, and transfusions were needed in several cases. The kinetics of immune activation following AAV-based GT (in persons with SMA or DMD) argues for TMA being an antibody-dependent event (classical pathway) amplified by the alternative complement pathway.[14] In those receiving prophylactic immune modulation with corticosteroids plus rituximab and sirolimus to prevent anti-AAV antibody formation, there was little change in immunoglobulins (Ig) IgM or IgG, and minimal complement activation. In contrast, in participants receiving corticosteroids only, a rapid increase in IgM and IgG and in D-dimer, a decline in platelet count, and both classical and alternative complement pathway activation indicative of TMA occurred.[14] Direct central nervous system (CNS) toxicity has been reported in one trial for late infantile Batten disease.[15] Eighteen months after intracerebral administration (via catheter injections) to trial participants of 2.9x10^{11} vg/kg of AAV.rh10-hCLN2 into 12 sites within the brain parenchyma, T2 abnormalities consistent with localized inflammation and edema at the site of injection were detected by magnetic resonance imaging. One participant in a trial on amyotrophic lateral sclerosis (ALS) reported significant neurological deficits and burning pains 3-4 weeks after intrathecal (IT) delivery of 4.2x10^{14} vg of an AAV.rh10 vector expressing superoxide dismutase 1. At autopsy 15.6 months after vector infusion, treatment-associated toxicity within the peripheral nervous system (PNS) and neuronal loss were observed in the dorsal root ganglion (DRG). Similar neuronal findings, in the absence of signs of toxicity or local inflammation, were also observed at pathology examination 8 months after vector administration in a patient following IT AAV delivery to the cerebrospinal fluid (CSF) in a clinical trial targeting giant

Table 1. Major adverse events reported in adeno-associated virus-based gene therapy clinical studies.°

Organs / systems involved, signs and symptoms	Disease / NCT/ sponsor	Clinical study / vector	AAV serotype / dose	Clinical summary, cases reported, N
Liver / biliary hallmarks	HA§§ 03392974 Biomarin	BMN-270 valoctogene roxaparvovec	AAV5, 600x10^{11} vg/kg	115/134 subjects, sustained transaminase elevation in some cases (>6 months)[101]
	HA§§ 04370054 Pfizer/Sangamo	SB-525 giroctocogene fitelparvovec	AAV5, 300x10^{11} vg/kg	5/11 subjects[102]
	HA§§ 03003533 Spark	SPK-8011	LK03§, 5-20x10^{11} vg/kg	7/18 subjects[103]
	HB§§ 02396342 UniQure	AMT-061	AAV5, 200x10^{11} vg/kg	Von Drygalski *et al.*[104]
	HB§§ 03861273 Pfizer/Spark	SPK9001 fidanocogene elaparvovec	SPK100, 5x10^{11} vg/kg	2/10 subjects[78]
	HB§§ 05164471 Freeline	FLT180a	AAVS3§ 4.5-12 x10^{11} vg/kg	4/10 subjects[105]
	SMA 03306277 Avexis/Novartis	Zolgensma	AAV9, 1100 x10^{11} vg/kg	Hepatocellular (in 90/100 trial subjects); liver failure responsive to steroids (2 / >1,500 patients), 2 deaths. ^[106,107]
	XLMTM 03199469 Astellas	AT132 resamirigene bilparvovec	AAV8, 1300-3500 x10^{11} vg/kg	Hepatobiliary; deaths: 1/6 in 1.3x10^{14} vg/kg and 3/17 in 3.5x10^{14} vg/kg cohorts.[11,26,108,109]
Thrombotic microangiopathy	SMA 03306277 Avexis/Novartis	Zolgensma	AAV9, 1100 x10^{11} vg/kg	Hemodialysis ± eculizumab (needed in some patients), death in 1 of >1,500 patients treated.[9,13]
	DMD 03368742 SolidBio	SGT-001	AAV9, 2000 x10^{11} vg/kg	2/6, managed with eculizumab and/or hemodialysis; no events after reducing empty AAV capsid dose.[9]
	DMD 06939926 Pfizer	PF-06939926	AAV9, 3000 x10^{11} vg/kg	2/9, responsive to eculizumab and/or hemodialysis.[9]
	Dannon 03882437 RP-A501 Rocket	RP-A501	AAV9, 1100 x10^{11} vg/kg	1/2, managed with hemodialysis and/or eculizumab.[110]
	MMA 04581785 LogicBio® Ther.	LB-001§§§ SUNRISE	AAV-LK03, two doses: LK03§ 500 x 10^{11} vg/kg LK03§ 1000 x 10^{11} vg/kg	2 patients treated with LB-001 experienced TMA in this phase I/II trial. Both cases were resolved within weeks. LogicBio has adjusted the protocol, added frequent testing and the provision of immune modulation if TMA is detected.[111]
Central nervous system toxicology	Late infantile Batten disease 01161576 Cornell	AAVrh10- CUCLN2	rh10, 2.85-9x10^{11} vg (IP, total dose)	1/2, T2 hyperintensity, MRI brain.[9,15,107,112]
Peripheral nervous system toxicology	ALS Nationwide	AAV miR-SOD1	rh10, 4200x10^{11} vg (IT, total dose) + methyl-prednisone bolus then daily prednisone	1/2. Patient 1: clinical symptoms meningo-radiculitis, DRG inflammation on MRI, and death. Patient 2: methylprednisone + rituximab pre-treatment + prednisone + tacrolimus post treatment; complications avoided.[16]
	GAN 02362438 Taysha Gene Therapies, Inc	AAV9	350 x10^{11} vg/kg (IT, total dose)	No local signs of inflammation or clinical symptoms of toxicity, severe neurological loss in DRG at autopsy 8 months after vector administration, death.[109,113]

Continued on following page.

Venous thrombosis*	HA 04370054 Pfizer/Sangamo	SB-525 giroctocogene fitelparvovec	rAAV2/6, 300 x10^{11} vg/kg	1/11, venous thrombosis, supratherapeutic expression.[102,114]
	HB 05164471 Freeline	FLT180a	AAVS3§ 4.5-12 x10^{11} g/kg	1/10 venous thrombosis, supratherapeutic expression.[105]
Myocarditis**	DMD 06939926 Pfizer	PF-06939926 fordadistrogene movoparvopvec	AAV9, 3000 x10^{11} vg/kg	3/16 subjects, 1 death (suspected immune response to transgene-derived protein; study completed).[115]

AAV: adeno-associated virus; ALS: amyotrophic lateral sclerosis; DRG: dorsal root ganglion; DMD: Duchenne muscular dystrophy; GAN: giant axonal neuropathy; HA: hemophilia A; HB: hemophilia B; IP: intraparenchimal; IT: intrathecal; MMA: methylmalonic acidemia; MRI: magnetic resonance imaging; N: number; NCT: National Clinical Trial identifier; SMA: spinal muscular atrophy; TMA: thrombotic microangiopathy; vg: vector genomes; XLMTM: X-linked myotubular myopathy. §AAV3-derived. §§Asymptomatic hepatocellular. §§§LB-001 is an investigational genome editing therapy for early intervention in MMA that uses GeneRide, a technology employing homologous recombination to enable precise genome editing without the need for exogenous nucleases and promoters. LB-001 is designed to non-disruptively insert a corrective copy of the methylmalonyl-CoA mutase (MMUT) gene into the albumin locus to drive lifelong therapeutic levels of MMUT expression in the liver, the main site of MMUT expression and activity. LB-001 is delivered to hepatocytes intravenously via rAAV-LK03. *Enrolment on hold. **Transgene toxicity. ^Acute liver failure is part of a black box warning on Zolgensma's US label. A recent safety alert was triggered by 2 death cases that Novartis reported in August 2023. Two children in Russia and Kazakhstan died of acute liver failure after receiving Zolgensma. Both patients had received corticosteroid to reverse liver damage. The European Medicine Agency's pharmacovigilance risk assessment committee planned to have a "Dear Doctor" letter distributed to inform physicians of Zolgensma's fatal events. The letter will also include advice that treating physicians adjust corticosteroid regimen and consult a pediatric liver disease specialist if patients do not respond adequately to initial corticosteroid treatment. No new liver failure deaths have been reported with Zolgensma since August 2023. °Modified from Samelson-Jones *et al.*[9]

axonal neuropathy (GAN). Participants received 3.5x10^{13} vg of an AAV9 vector.[16,17]

In a scenario of the resurgence of non-viral gene transfer approaches,[18] significant improvement (i.e., better cassette engineering) is of utmost value for AAV vectors (Table 2). To this end, studies in non-human primates (NHP) help to recognize underlying determinants and mechanisms of PNS[19,20] and CNS[21,22] toxicity.

Preclinical studies

Factors associated with neurotoxicity in adeno-associated virus-based gene therapies

Route of administration

The blood-brain barrier controls the transit of drugs, immune cells, pathogens, and AAV vectors into neurons. AAV-associated neurotoxicity is more often observed in NHP receiving AAV via intra-CSF injection than via IV injection.[23] However, it also occurs after systemic delivery of higher vector doses (>10^{13} vg/kg).[24]

Vector dose and delivery

Localized delivery of >10^{9} vg/kg of brain tissue exposes neurons to more AAV/cell and to local neurotoxicity and inflammation at the injection site.[19,24-26] Systemic delivery of higher vector doses (>10^{13} vg/kg) exposes more neurons to AAV and triggers extensive toxicity in the nervous system.[24]

Capsid

By mediating cell binding and virus uptake, capsids directly influence tropism for neurons. Although some capsids appear to be less neurotoxic than others,[27] all tested neurotropic capsid serotypes were comparable to each other in a meta-analysis that considered the possibility for some AAV (e.g., AAV9) to transduce neurons better than others (e.g., AAV2).[23]

Inverted terminal repeats

Inverted-terminal repeats (ITR) are critical elements for AAV genome rescue, replication, packaging, and vector persistence.[25] In animal models, ITR-initiated aberrant transcription is linked to toxicity through the deregulated production of vector-derived mRNA and/or expression of toxic transgenes (or via the production of RNA produced from cross-packaged AAV packaging plasmids).[21,28] CpG islands are short, predominantly unmethylated, interspersed DNA sequences, equipped to regulate local chromatin structure gene activity. Due to their unique DNA sequence composition, silencing these functional promoters of transcription initiation is achieved through dense CpG methylation. In addition to toxicity for the DRG, preclinical data on CpG in the vector cassettes also suggest directions to understand why some vectors respond to steroids while others do not. Unmethylated CpG motifs trigger pro-inflammatory response via toll-like receptor9 (TLR9)-mediated recognition (innate immune sensing). Indeed, vectors depleted of CpG motifs minimize or circumvent an AAV capsid immune response.[29] The loss of transgene expression in an AAV8-based GT trial for HB (BAX335; *clinicaltrials.gov 01687608*) has been credited with stimulating innate immune responses, embracing the effect of CpG oligodeoxynucleotides introduced into the BAX 335 coding sequence by codon optimization.[30] The lack of effect of steroids in this study calls for the innate immune stimulatory effect of CpG motifs enriched within the vector cassette.

Transgene

Vector-delivered transgene products can be directly toxic

Table 2. Next-generation strategies in adeno-associated virus-based gene therapy: improved cassette engineering.*

Vector targeting	AAV capsid variants with improved tropism for the target tissue (capsid libraries)
	Lowering doses to decrease treatment-associated [neuro]toxicity
Regulating transgene expression	Regulatory elements and transgene expression
	Limiting transgene expression within unwanted cell types
	Limiting transcription to specific cell types
Innate sensing of AAV vector	Reducing CpG motifs within the AAV ITR or the vector backbone
	Limiting innate vector recognition
Ad hoc investigation Mechanisms of transgene expression, loss, and cell toxicity	Response of the patient's immune system to the capsid or the transgene
	Protein- or transgene- derived mRNA overexpression and (neuro)toxicity
	Clearance of transgene proteins: determinants
	The UPR pathway beyond neurotoxicity

AAV: adeno-associated virus; ITR: inverted-terminal repeats; UPR: unfolded protein response. *Suggested further reading.[116-118]

(enhancing cell death in transduced cells), or indirectly toxic (mediating immune responses that target transduced cells for death). Critical factors for such events include: type of transgenes delivered (foreign or self, foreign transgenes often being more neurotoxic than others), the AAV serotypes used, and levels of transgene expressed.[25] Some transgenes are not toxic in all species.

Promoter

Use of strong ubiquitous promoters is associated with neurotoxicity in NHP.[25] High transcription in AAV-transduced cells leads to high levels of mRNA and/or transgene, both triggering toxic events.[23] Whether this information is relevant in humans is still unclear. While some promoters of transgene expression are not toxic in all species,[31] vectors expressing foreign promoters (e.g., CAG, CMV, CBh, CB7) are directly toxic and immunogenic in most preclinical models.[32,33] For example, NHP given AAV vectors containing the CAG promoter had higher levels of neurotoxicity.[23] The safety of ubiquitous promoters had first been questioned by a study describing the development of hepatocellular carcinoma (HCC) in mice after systemic delivery of AAV GT vector for treatment of mucopolysaccharidosis type VII.[34] In a subset of tumors, AAV integrations were tightly clustered in the RNA imprinted and accumulated in nucleus (*Rian*) locus on chromosome 12 in the treated mice.[34] This genomic region encodes a variety of regulatory RNA, including microRNA.[35] The aberrant expression of proximal small non-coding regulatory RNA induced by AAV vector integration was intended as a mechanism for carcinogenesis.[34] HCC has also been documented in mice with different inborn errors of metabolism several months after neonatal AAV injections, and associated with vector integration and overexpression of microRNA-341 proximal to the RNA imprinted and accumulated in nucleus (*Rian*) locus.[36] In this study, the HCC risk correlated with vector dose and degree of cellular division, and was abolished by a hepatocyte-specific promoter. That said, genome microRNA-341 is missing from the genomes of larger animals (e.g., rabbits, cats, dogs, NHP).[37]

Regulatory elements

Depending on the transgene employed, elements increasing transcription and translation (e.g., the poly A signal) enhance production and toxicity of some transgenes in NHP.[38] Elements increasing transgene persistence or regulating transcription are often integrated into AAV vector genomes.[39,40] For some transgenes, elements regulating transcription (e.g., the tetracycline-controlled trans activator [tTa] and reverse tTa [rtTa]), impact toxicity[38] by removing immunogenic AAV-transduced cells.[41] These elements are not used in humans.

Impurities in adeno-associated virus vector stocks

Defective/empty capsids, residual producer cell components, serum or helper virus proteins, cross-packaged DNA from AAV packaging plasmids or helper viruses, and bacterial endotoxin are all contributing factors to neurotoxicity in NHP.[42-44] Good Medical Practice (GMP) grade vectors should be used to provide information from preclinical studies for exploring use in humans.

Mechanisms of adeno-associated virus neurotixicity

Adaptive and innate immunity

In spite of the evidence of neuroinflammatory responses to AAV-mediated gene therapies (T-cell, and mononuclear cell infiltration of sensory nerve and ganglia),[45] NHP receiving steroids or immunosuppressive therapy still display neurotoxicity despite blunted vector and transgene-specific immune responses.[24,46,47] Conversely, when CpG motifs are reduced in the vector backbone, less innate immune sensing occurs,[44] and transgene and vector-specific T-cell responses are reduced.[29,30,48-50]

Protein-folding overload

Nascent proteins are folded and secreted in the endoplasmic reticulum (ER). ER function overload induced by a greater demand for protein folding (or the accumulation of unfolded or misfolded proteins) leads to the unfolded protein response (UPR), a mechanism that detects the conformity of protein folding in the ER lumen.[51] The UPR pathway surveys the ER

and transfers information on protein folding status to the nucleus and cytosol to adjust the protein folding capacity of the cell or to induce apoptosis when chronic damage takes place.[52] Cell death caused by stress-induced apoptosis following activation of the UPR pathway in cells expressing the most transgene has been hypothesized to explain DRG toxicities in NHP.[24,53,54] Indeed, neuronal degeneration occurs preferentially in cells with the highest transgene expression. It is presently unclear whether, rather than the capsid or vector DNA,[55] overexpression of the transgene-derived mRNA or the protein mediates AAV-associated neurotoxicity.[25] Neither has the role of stress-induced apoptosis in explaining the loss of FVIII expression been thoroughly elucidated.

Aligning the interests of the healthcare system with the responsibilities of the pharmaceutical industry

Standardizing vector production and potency, and assuring quality control of large-scale vector manufacture are major determinants of product costs. Additional costs and challenges in AAV-based GT arise from the need for tests to measure outcome to be designed, verified, and validated as *ad hoc* endpoints to quantify clinical effectiveness when delivering a treatment to patients with diseases that have so far been incurable.[56] Further requirements emerge from the 5-year experience with voretigene neparvovec-rzyl GT for Leber congenital amaurosis.[57]

1) Extending collaboration to multidisciplinary teams that are equipped for treatment intervention, and offer access to baseline and follow-up visits (Centers of Excellence). A pre-requisite for such promotion is the availability of standard operating procedure manuals to set up training programs for medical teams to standardize AAV vector genome delivery.

2) Persons with chronic diseases, (e.g., hemophilia) develop high confidence in their referring centers and may not wish to move to a new one. At the moment, expertise in GT is available in only a very few centers. The active role of the pharmaceutical industry is critical to ensure interaction between referral and treatment centers, and guarantee health equity.

3) It is presently unclear whether the benefit of voretigene neparvovec-rzyr is greater when the treatment is performed in younger rather than in older individuals. Publishing both the positive and negative data obtained in the post-marketing phase of development, and providing original data avoid repeating unsuccessful studies.

4) Social media offer a privileged approach to sharing global information on the efficacy and safety of GT, and the latest results and technologies. This interface also helps patients, families and associations to contact reference centers, and to set up their own education sessions with scientists and clinicians.[57] An additional benefit is that some people with ultra-rare genetic disorders may volunteer to participate in natural history studies, and in the evaluation of novel outcome measures. The dissemination of unofficial data before critical review and publication, and the risk of misinformation is a downside of the social media interface.[57] To ensure that correct information is disseminated, all posts should include pictures of slides or reference specific abstract presentations, papers, etc.

The adenosine deaminase-severe combined immuno-deficiency (ADA-SCID) GT model (γ-retroviral transduction of hematopoietic stem cells for treatment of ADA deficiency causing SCID) extends to *ex vivo* GT strategies the need for comprehensive standard operating procedures, and training program networks for qualified medical teams. The model also emphasizes the need for the pharmaceutical industry to take on new responsibilities in order to improve global access to ATMP.[58] Making GT accessible to patients worldwide implies guaranteeing sufficient manufacturing capacity for the needs of expanded applications, to safeguard post-marketing supplies, and to standardize process development across different countries (Table 3).

Patient eligibility criteria and clinical endpoint definition

In diseases with low patient numbers, and medical regimens marked by significant non-adherence due to the burden of treatment (e.g., severe hemophilia), randomized and even case-control studies are usually unfeasible.[59] Under these conditions, knowledge of the patient's familial and clinical history is key for patient eligibility criteria, to define unequivocal endpoints (i.e., for clear-cut results by supplying the missing / defective gene), and for GT success.[6] Examples of approaches to clarify this are presented here.

a) Fatty liver syndrome is emerging as a common source of chronic liver disease and of hepatic fibrosis.[60,61] Identifying and evaluating a fatty liver is critical to the success of AAV-based liver-directed GT in persons with haemophilia (PWH). Biomarkers to stratify the stage of the fatty liver syndrome, predict long-term outcomes, and monitor responses to diet / drugs are urgently needed.

b) Supraphysiologic Factor VIII (FVIII):C levels are independent risk-factors (odds ratio range: 8.8-21.3) of venous thrombosis, mainly in the elderly.[62] Because of transgene-derived circulating FVIII activity levels >150% (upper limit of normal [ULN]) in some PWH who had undergone AAV-based GT, the sponsor (Sangamo/Pfizer) paused the phase III C3731003 FVIII gene therapy study aimed at evaluating the clinical efficacy and safety of a single infusion of PF-07055480/giroctocogene fitelparvovec (rAAV2/6 SB-525 vector), amended the protocol, and implemented risk minimization measures together with the external Data Monitoring Committee. During the pause, a thrombotic event occurred in an infused PWH with a recent significant reduction in physical activity, and upper normal

Table 3. Extensive requirements for the pharmaceutical industry.

GT treatments for all: make ATMP accessible to patients worldwide
Decrease the therapeutic dose: newer vectors with higher efficiency and safety of gene transfer
Standardize process development across different countries
Guarantee adequate manufacturing capacity for the needs of expanded applications
Guarantee worldwide post-marketing supplies: standard operating procedures, training programs, qualified treatment centers
Maintain sustainability and cost-effectiveness of personalized therapies
Address the lack of robust data on safety: ensure pharmacovigilance and long-term follow-up monitoring (safety records grounded on large data collections and focused on carcinogenesis, toxicity, and germline transmission of donated gene sequences)
Address the uncertain durability of the GT response: gather robust data on durability from long-term, potentially life-long, pivotal studies in settings without rapid transgene decline (emphasis on steroid use [dose, duration], immune response, and durability of the GT response in the reports)
Improve collaborations with the Academy (e.g., the ADA-SCID GT model)
Developing countries: commercialization, pricing, reimbursement, and access to one-off treatments for rare genetic disorders
Define minimal criteria needed to clinically administer GT
Improve treatment standards and product availability to the level of higher-income countries
Tailored approaches to drug pricing for approved GT treatments
Maintain costs similar/cheaper than the socio-economic costs of current lifetime treatments*
Payment systems based upon cost of goods (other than value-based pricing)

ADA-SCID: adenosine deaminase-severe combined immuno-deficiency; ATMP: advanced therapeutic medicinal products; GT: gene therapy. *Varied pricing in different countries.

FVIII activity levels.[63] High, stable (up to 1 year) expression levels of FIX (24-168 IU/dL at 3 weeks), were detected after GT in the phase I B-AMAZE study using FLT180a, a AAVS3 capsid carrying a *F9* variant with a gain-of-function mutation. A participant with a high FIX expression (>200 IU/dL) developed a thrombotic occlusion of an arteriovenous fistula (Table 1). Whether to strive for 'normal levels' rather than for 'therapeutic levels' of transgene protein is an open issue in hemophilia GT.[64,65]

c) Long-term data from patients with transfusion-dependent β-thalassemia who have undergone GT extend the impact of these examples to an *ex vivo* lentivirus-based approach also employed in children,[66] and provide basic information and hints to optimize patient access to AAV-based GT in hemophilia (Table 4). Indeed, global evaluation of organ function (e.g., liver, heart, lungs) is critical beyond the age for optimal patient selection and GT success in this clinical setting,[66] and persistently high transgene level expression is a key improvement endpoint to prevent complications of the disease that may otherwise occur later in life.[67] Biomarkers that help to predict long-term outcomes and complications should be identified.

Advancing the use of adeno-associated virus-based gene therapies: contexts and prospects

Despite the tenet that AAV-based GT can vary in design and endpoints, and that informative outcomes may differ accordingly,[68] clinical and preclinical data in the area provide broad conclusions and directions to be followed for advancing AAV-based GT.

In the HOPE-B trial (*clinicaltrials.gov 03569891*), 54 adult males with HB were enrolled regardless of a history of hepatitis B virus or hepatitis C virus (HVC) infection.[69] Participants received a single IV dose of etranacogene dezaparvovec ($2x10^{13}$ gc/kg), comprising a liver-directed rAAV5 vector containing a codon-optimized Padua-variant human FIX transgene, and a liver-selective promoter. Molecular and vector integration analyses of a case of hepatocellular carcinoma (HCC) one year after GT in a participant with a long-standing history of HCV infection, established no relationship with rAAV administration and provided a model for exploring malignancy in participants in GT studies with integrating vectors.

Using a similar approach, no relationship has been reported for the tonsil cancer in a participant in the BAX-335 trial.[30] Finally, in 2 patients who had been infused with valoctocogene roxaparvovec three and five years before for severe hemophilia A (HA) and who developed a salivary gland carcinoma and a B-cell acute lymphoblastic leukemia, respectively, whole genome sequencing analysis led the trial's Data Monitoring Committee to argue against such malignancies being related to GT.[70]

Pre-clinical data confirm and extend the conclusions of such studies in humans. Low-frequency AAV integration mostly in sites of active transcription has been documented in dogs, together with AAV integration and clonal expansion of cells with insertions near genes that are potentially associated with growth control.[71] However, none of the dogs showed overt nodule formation or transformation (or abnormal liver function related to AAV administration) in

Table 4. Clinical product development, explanatory endpoints: investigational gene therapy.

Short-term goals	
Endpoints	**Suggested strategy**
Target cells/tissue (emphasis on liver cells)	Better understanding of the pathophysiology (and turnover)
Tropism of AAV serotypes	Enabling detection of differences
Outcomes, safety, and durability of GT	The roles of manufacturing, treatment-, and patient-related parameters
Eluding/blocking AAV-associated toxicity	An understanding of TLR/CpG recognition, and miRNA binding sequences
Long-term goals	
Endpoints	**Suggested strategy**
Transgene expression: extending stability and durability	? Lentiviral vectors encoding the transgene
	? Striving for 'normal levels' rather than for 'therapeutic levels' of the circulating protein
Expanding indications for GT	Persons with pre-existing high anti-AAV neutralizing antibodies
	Children: transgene expression diluted and lost over time
	Beyond the upper and lower age limits for patient selection
Quality of life variables	Improved physical, social and mental health
	Freedom from medications
	Caution, e.g., when using the term "curative"

AAV: adeno-associated virus; GT: gene therapy; TLR: toll-like receptor.

the ten years after transgene delivery. Intravenous dosing of AAV8 and AAVrh10 vectors argues for AAV-mediated transgene expression in NHP hepatocytes as occurring in a short-lived, high expression from episomal genomes (the first 90 days), followed by a lower stable expression, likely from integrated vectors.[72] Single nuclear domains of vector DNA were documented in >10% of hepatocytes that persisted despite the loss of transgene expression. Genomic integration of vector sequences was detected in 1/100 cells at broadly distributed loci that were not in proximity to genes associated with HCC. Overall, despite the fact that genome microRNA-341 (the nucleus of the rAAV HCC site in mice) is missing from the genomes of large animals,[37] the risk of genotoxicity leading to cancer remains a major safety concern of high-dose AAV vector infusion. Conclusive information is expected from data from infants receiving high systemic AAV vector doses with a ubiquitous promoter (e.g., Zolgensma®).[9] Because of the rapid growth of the liver and the high rates of cellular division, such a juvenile setting resembles to a certain extent the risk of genotoxicity active in mice following AAV administration.[36]

Based on the data from the trials in patients with lipoprotein-lipase (LPL) deficiency receiving alipogene tiparvovec (Glybera™), the EMA argued against unjustified prophylactic administration of immune-suppressive agents in AAV-based GT.[73] Indeed, muscle biopsy specimens showed ongoing transgene expression in subjects treated in the phase I trial (in which no immune suppression was used), in the second trial (where cyclosporine and mycophenolate mofetil were included), and in the third trial, with the addition of a high-dose injection of steroids. Apart from the concerted action of both the adaptive[8,74] and the innate[75-77] arms of the immune system, stress-induced apoptosis could help assess the level of supportive data beyond DRG toxicity in GT. Physiologically, hepatocytes synthesize FIX, and endothelial cells of liver sinusoids and other tissue-specific endothelial cells synthesize FVIII.[63] Like in the nervous system,[24] transgene expression (and cell death) is limited to a subset of liver cells with the highest transgene expression in AAV-based GT in hemophilia. Together with mild, asymptomatic transient increases in ALT levels matched with a detectable corticosteroid-controlled anti-AAV capsid T-cell response,[74,78,79] ALT levels 1.5- to 2-fold higher than the upper normal limit may (e.g., in HB) or may not (e.g., in HA) be associated with hepatocyte loss.[30,80] In some cases, increases in ALT level occur without a capsid response,[81] and capsid response and increased ALT levels may be independent, but parallel events.[74,82] An increase in ALT levels was independent of the loss of FVIII activity or a T-cell immune response to capsid peptides in most cases of the BioMarin / Roctavian phase I/II study (*clinicaltrials.gov 02576795*).[80,83]

Data on the role of patient-, manufacturing-, and treatment-related parameters are emerging from the understanding of the mechanisms of the loss of transgene expression.[83,84] The uncertain durability of the GT response argues for pushback by the regulators for life-long follow-up data,[7] and for long-term pivotal studies in settings without rapid transgene decline, both in the presence and in the absence of steroid use.

Cell surface attachment (the first step in the delivery, on the part of AAV, of their cargo to the target cells) occurs via directions targeted to increase the chance of engaging

with transmembrane receptor proteins to increase AAV entry and internalization. The expression of specific cell surface glycans impacts early binding and internalization of AAV and AAV tropism. Attachment to cell membrane heparan sulphate proteoglycan is the initial step in the interaction of the AAV2 serotype with the target cell. Other glycans behave similarly to other AAV serotypes (e.g., sialic acids for AAV5, -1, -6 and -4, and galactose for AAV9).[85] Together with these 'primary receptors', still unidentified 'co-receptors' are thought to govern cellular tropism and internalization. Within their antigens or the genetic material, viral vectors carry pathogen-associated molecular patterns (PAMP).[86] Being absent in mammals, PAMP are perceived as threats by pathogen recognition receptors (PRR) located on the cell surface, within endosomes or the cytosol. Expression of several PRR (e.g., TLR) is cell-type specific: TLR2 is expressed by macrophages but not by dendritic cells, while TLR3 has an opposite pattern of expression.[87] PAMP binding to a PRR initiates a signaling cascade to activate transcription factors, e.g., nuclear factor κβ, or interferon regulatory factors 3 and 7, that ultimately lead to cytokine and chemokine formation.[88] Relationships between cell interaction with different AAV subtypes and toxicity in AAV-based GT should be fully explored.

Studies in patients with congestive heart failure emphasize the lack of animal models resembling the heterogeneity of the clinical population evaluated regarding different etiology and disease progression, and argue for tailoring vector dosing and administration to phenotypes mimicking distinct phases and mechanisms of disease (e.g., ischemic, hypertensive, etc).[89] Knowledge of the natural history of the disease is critical for clear and unambiguous evidence of treatment effects (i.e., clinical improvement endpoints). Due to the rarity of the inherited deficiency (prevalence: 1 per million live births), the high costs to the patient, and the expense to the company of maintaining therapeutic readiness, Glybera™ was withdrawn from the market in 2018. At that time, only 31 people in the world had been treated with this ATMP. On September 2023, to prevent any problems for patients due to the risk of stopping sales in Europe, the EMA authorized the non-profit association TELETHON to produce and commercialize Strimvelis,™ the first GT product for transfusion-dependent severe β-thalassemia.[67] Because of the costs and technical complexity, it is unlikely that current protocols will be applicable where the greatest demand for AAV-based GT for a monogenic disease lies.[90] Bridging the gap between the companies developing these therapeutic approaches, and their availability to the patients that may benefit from them the most is a complex task. In 2020, the Global Gene Therapy Initiative was formed to tackle the barriers to inclusion of several low- and middle-income countries (LMIC) in GT development.[90] This group has set a goal of introducing two phase I GT trials in two LMIC, Uganda and India, by 2024.

Perspectives: further lessons to be learned

Research and development for ATMP continue to grow at a fast rate in the EU and the US to help in designing future AAV vectors. Several products are undergoing rapid clinical development,[90] and time points to allow for critical interventions impacting the safety and efficacy of systemic GT have been identified.[14] Although the FDA requires all patients receiving any lentiviral vector to be followed for 15 years, none of the patients treated with newer vectors developed any leukemia or myelodysplastic syndrome.[66] While *ad hoc* data are needed to dissipate any remaining concerns, gene editing[91] and antisense approaches[92] are expected to complement gene augmentation for permanent solutions of unsolved issues of AAV-based GT. Proof of concept of F9 gene editing has been provided in NHP models.[93] A hematopoietic stem cell transplantation protocol has been registered that incorporates a lentiviral vector encoding the highly expressing FVIII transgene ET3 (Study ET3-201, Expression Therapeutics) to achieve stable FVIII expression for the treatment of severe HA. In growing children, AAV-mediated transgene expression is diluted and theoretically lost over time. A gene-editing program that uses a CRISPR/Cas9-based *in vivo* genome editing and enables permanent chromosomal integration of a modified human B-domain-deleted FVIII at the albumin locus (to prevent the loss of AAV vector due to hepatocyte proliferation) in liver cells is currently being pursued (ASC Therapeutics) in order to advance step by step towards durable treatment options in young persons with HA.[91,94] Obviously, additional lessons could be learned. An acute respiratory distress syndrome (ARDS) due to an innate immune reaction occurred in a 27-year old patient with DMD treated with high-dose GT ($1x10^{14}$ vg/kg) with an rAAV9 serotype vector containing dSaCas9 (i.e., 'dead' *Staphylococcus aureus* Cas9, in which the Cas9 nuclease activity has been inactivated) fused to VP64. This transgene was designed to up-regulate cortical dystrophin as a custom CRISPR-transactivator therapy. Prior to the GT, the patient received prophylactic immune-suppression (rituximab, glucocorticoids, sirolimus), and underwent infectious disease evaluation. Six days after treatment, mild cardiac dysfunction and pericardial effusion developed, followed by ARDS and cardiac arrest. Pathological examination showed severe diffuse alveolar damage and unexpectedly elevated levels of vector genome in the lungs. There was no evidence of anti-AAV9 antibodies or effector T-cell reactivity in the organs. ARDS is not common in association with AAV-based GT, and other persons treated with the same dose of the rAAV9 vector did not experience this toxic effect. Both host factors and inherent properties of the vector (including requirements for transgene expression) may have contributed

to the outcome; both should be thoroughly explored in order to improve GT as a clinical discipline.[95]

Whether maintaining cost-effectiveness of personalized therapies and long-term monitoring and pharmacovigilance will suffice for the global success of the GT initiative should be assessed. It is unclear to what extent GT will help achieve health equity.[96] Together with missing or late diagnosis and the lack of regular medical reviews, mortality due to severe bleeding (e.g., intracerebral bleeding) or to bleeding becoming severe (e.g., bleeding associated with circumcision in the absence of appropriate measures) is assumed to explain the abnormally low number of hemophilia patients (expected *vs.* observed) in Sub-Saharan Africa.[97] Neither has the impact of GT adoption on de-medicalization been thoroughly explored so far.[98] However, the mounting recognition of the value of GT by clinicians, patients, the industry and policymakers reflects the emergence of a field that is estimated to leave a major imprint on medical practice. These are the early days for AAV-based GT, a more complex 'drug' than small molecule and well characterized protein drugs. With the refinement of vector manufacturing, and newer economic models to improve global access, GT is expected to achieve better safety and durability outcomes, and provide newer curative options to clinical medicine.[57] When the term "gene therapy" disappears from the medical lexicon, we will know that safe gene-based medicines have become the norm, and that patients are receiving maximum benefit from a tailored, molecularly-targeted approach.[99] Major achievements may take longer than what the original excitement had led us to believe.[100]

Disclosures

GDM is speaker or a member of a speaker bureau for BioMarin, Bayer, CSL Behring, Roche, Takeda, Viatris Pharmaceuticals, and consultant or ad-hoc speaker / consultant for BioMarin, Bayer, Pfizer, Takeda, Viatris Pharmaceuticals. GC has received grant / research support from CSL Behring, Pfizer, Sobi, is a consultant for Ablynx, Alexion, Bayer, Takeda, CSL Behring, Novo Nordisk, Pfizer, Roche, Sanofi, SOBI, uniQure (membership on an entity's Board of Directors or advisory committees), and sits on the Speaker Bureau of Bayer, Bioviiix, Roche, Sobi, Grifols, Novo Nordick, Werfen, Kedrion. FP is a speaker or a member of a speaker bureau for Grifols and Roche, and sits on advisory boards for Sanofi, SOBI, Takeda, Roche, and BioMarin. WM: Bayer, Biomarin, Biotest, CSL Behring, Chugai, Freeline, LFB, Novo Nordisk, Octapharma, Pfizer, Regeneron, Roche, Sanofi, Sigilon, Sobi, Takeda / Shire, and uniQure.

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